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Inhibitors of Nonhousekeeping Functions of the Apicoplast Defy Delayed Death in *Plasmodium falciparum*▽

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Targeting of apicoplast replication and protein synthesis in the apicomplexan *Toxoplasma gondii* has conventionally been associated with the typical "delayed death" phenotype, characterized by the death of parasites only in the generation following drug intervention. We demonstrate that antibiotics like clindamycin, chloramphenicol, and tetracycline, inhibitors of prokaryotic protein synthesis, invoke the delayed death phenotype in *Plasmodium falciparum*, too, as evident from a specific reduction of apicoplast genome copy number. Interestingly, however, molecules like triclosan, cerulenin, fops, and NAS-91, inhibitors of the recently discovered fatty acid synthesis pathway, and succinyl acetone, an inhibitor of heme biosynthesis that operates in the apicoplast of the parasite, display rapid and striking parasiticidal effects. Our results draw a clear distinction between apicoplast functions per se and the apicoplast as the site of metabolic pathways, which are required for parasite survival, and thus subserve the development of novel antimalarial therapy.

The killer disease malaria continues to stalk millions of people in the tropical and subtropical terrains of the world (34). Caused by the apicomplexan protozoan parasite *Plasmodium*, malaria exacts a high mortality rate and is one of the biggest killers of children in the tropics. Moreover, several strains of the malaria parasite are currently resistant to chloroquine and other frontline antimalarial drugs. The situation clearly calls for the identification of new antimalarial drug targets.

The apicoplast is a four-membrane-bound relict plastid of endosymbiotic, eukaryotic algal ancestry in *Plasmodium* and other apicomplexans (12). The presence of the apicoplast and its indispensability to the parasite make it a potential Achilles' heel, thus offering great promise for combating malaria and other diseases caused by apicomplexans (5, 17, 35). The apicoplast houses prokaryotic machinery, presumably to replicate its circular 35-kb genome and to transcribe and translate the genes that it possesses. Consequently, ciprofloxacin, earlier reported to inhibit the prokaryotic DNA gyrase, and chloramphenicol, clindamycin, and other lincosamide antibiotics, which are believed to inhibit transpeptidation of prokaryotic protein synthesis, abrogate *Plasmodium* growth (3, 23, 24, 27). More importantly, the apicoplast is predicted to provide the microenvironment required for fatty acid synthesis, non-mevalonate isopentenyl diphosphate synthesis, and some of the reactions of the heme biosynthesis pathway, and given the cyanobacterial heritage of the apicoplast, many of the nucleus-encoded and apicoplast-targeted enzymes involved in these pathways are fundamentally different from those in their mammalian host counterparts, thereby making them potent drug targets (9, 28, 29). Inhibitors of these apicoplast resident metabolic pathways—triclosan (7), cerulenin (32), aryloxyphenoxypropionate herbicides (20), NAS-91 (25), succinyl acetone (15), and fosmidomycin (14)—have been demonstrated to kill *Plasmodium* (8, 14, 25, 28, 29, 32, 33).

Ciprofloxacin, clindamycin, and chloramphenicol invoke peculiar and distinctive kinetics of death termed the "delayed death" phenotype in *Toxoplasma gondii* (2). Treatment of *Toxoplasma* with these drugs does not affect the doubling frequency of these parasites in the first host cell; however, division is slowed upon subsequent invasion of a new host cell. Although this delayed death invoked by the effects of these drugs on apicoplast functions one generation following drug intervention is an interesting and intriguing biological phenomenon by itself, it is a severe limitation where clinical application is concerned. This is especially pertinent in malaria patients, in whom a single cycle of asexual reproduction in *Plasmodium falciparum* takes 48 h to complete and a delay of 48 h or more in treating malaria could have severe consequences for the patient.

The delayed death phenotype has been rationalized in *Toxoplasma* as the consequence of the generation of daughter cells devoid of an apicoplast due to the inability of the apicoplast to segregate following inhibition of an apicoplast function (2, 6). That being so, inhibition of the recently discovered type II fatty acid synthesis occurring in the apicoplast should lead to a similar fate. The antimicrobial biocide triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol], which targets the enoyl-acyl carrier protein (ACP) reductase of the type II fatty acid biosynthesis pathway, potently incapacitates fatty acid synthesis in this organelle. However, we had observed that triclosan abro-

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gates parasite growth rapidly (29). While this finding indicates a dichotomy in the mechanisms of action of inhibitors of apicoplast functions, it raises several questions as well. In particular, do antibiotics like clindamycin and chloramphenicol, earlier reported to invoke delayed death in *Toxoplasma*, invoke delayed death in *Plasmodium falciparum*, too? If they do, does triclosan bring about "rapid death" by virtue of inhibition of fatty acid synthesis per se? If this is true, then does inhibition of the other apicoplast nonhousekeeping metabolic pathways, such as heme biosynthesis, also invoke rapid death? We have endeavored to address these questions here.

MATERIALS AND METHODS

Materials. The culture medium components RPMI 1640 and HEPES, as well as the reagents, sorbitol, and dimethyl sulfoxide (DMSO), were purchased from Sigma. Triclosan 5000 was obtained from Kumar Organic Products Ltd., Bangalore, India, and its antibacterial effect was tested by using *Escherichia coli* Kan 91 cells prior to its use on *Plasmodium* culture. Acifluorfen and haloxyfops were procured from Dr. Ehrenstorfer Chemicals, GmbH. All other inhibitors were obtained from Sigma Chemicals. Inhibitor stocks were made directly either in RPMI 1640 or in DMSO. Stocks were prepared such that the final concentration of DMSO did not exceed 0.05% in the culture medium. Thiolactomycin was a kind gift from Laurent Kremer, France. *R*-Lipoic acid and *S*-lipoic acid were kind gifts of Mulchand Patel (SUNY at Buffalo).

Intraerythrocytic cultures of *Plasmodium falciparum*. For experiments requiring *Plasmodium* cultures, chloroquine-sensitive *P. falciparum* strain FCK2 (chloroquine sensitive; 50% inhibitory concentration [IC_{50}], 18 nM) was cultivated in type O-positive human erythrocytes in medium supplemented with type O-positive human serum by the candle jar method of Trager and Jenson (31). Cultures were synchronized by 5% sorbitol treatment (13), and parasites were observed for viability and changes in morphology by standard Giemsa staining.

Determination of death kinetics by microscopy. To monitor the effects of the various antimalarial compounds on the parasites by microscopy, red blood cells infected with parasites synchronized at the ring stage were cultured in 96-well plates (Nunc, Copenhagen, Denmark) at 10% hematocrit and at an initial parasitemia of ~3%, with a change of medium every 24 h. Inhibitors were added at the required concentrations. The inhibitor concentrations used in the experiments were based on previously published findings as well as by our own observations. All inhibitor additions were done in two sets. In the first set, inhibitor was present in the culture medium throughout the duration of the experiment, i.e., for 96 h. In the second set, inhibitor was present in the culture medium only up to 48 h. Every inhibitor concentration was tested in triplicate. A thin blood smear was prepared every 12 h, and the parasites were observed microscopically in Giemsa-stained smears. The percent parasitemia was calculated from the ratio of the number of infected red blood cells to the total number of red blood cells. Red blood cells were counted in at least 10 independent fields, each with approximately 200 cells, by using a light microscope (Olympus, Japan). Dead parasites within red blood cells could be differentiated from live cells by the absence of an intact membrane and/or the absence of a stained cytoplasm. Unhealthy parasites, such as those with vacuolation in the cytoplasm, were not counted as dead cells.

Determination of parasite growth by [^{3}H]hypoxanthine uptake growth inhibition assay (in vitro inhibitor susceptibility assay). Antimalarial compounds were tested in a cell-based in vitro inhibitor susceptibility assay to determine if they were capable of inhibiting *P. falciparum* growth. The semiautomated microdilution technique of Desjardins et al., which is based on [^{3}H]hypoxanthine uptake by parasite cultures, was used to assess the sensitivities of the parasites to the selected compounds (1). Briefly, synchronized parasites were cultured in 96-well plates (Nunc) at 2 to 3% hematocrit and at an initial parasitemia of 1 to 2% with various concentrations of inhibitors and with the addition of inhibitor in fresh medium every 24 h. All additions were done in duplicate. For every inhibitor tested, the MIC (MIC_{90}) and IC_{50} were determined in two sets. In the first set, parasites synchronized at the ring stage were cultured in the presence of [^{3}H]hypoxanthine and various concentrations of the particular inhibitor for 48 h. They were then harvested onto glass fiber filters by using a Nunc cell harvester, washed, and subjected to liquid scintillation counting (Hewlett-Packard). In the second set, parasites synchronized at the ring stage were cultured in the presence of various concentrations of inhibitor for the first 48 h. Subsequently, the cultures were incubated with [^{3}H]hypoxanthine for 48 h and harvested. IC_{50}s and MIC_{90}s were calculated from plots of the relative percent parasitemia versus the log concentration of inhibitor; the concentrations were fitted by nonlinear regression analysis by using Sigma Plot 2000 software. The relative percent parasitemia was calculated as the percent parasitemia of the parasites under treatment and by considering that of untreated parasites to be 100%.

Determination of change in plastid genome/nuclear genome ratio by quantitative dot blot hybridization. Parasites were cultured in human red blood cells at 10% hematocrit with or without inhibitor. Parasites were isolated from infected red blood cells by lysis with 0.15% saponin (Sigma) in phosphate-buffered saline (pH 7.4). Total DNA (~2 μg) was extracted from the parasite pellets of control and drug-treated parasite cultures by treatment with proteinase K (Sigma), RNase, and phenol-chloroform extraction and ethanol precipitation. Total DNA was blotted manually, under alkaline transfer conditions, onto a nylon membrane (Hybond N+; Amersham Biosciences). The genes *eftu* (the apicoplast gene that codes for elongation factor Tu) and *fabI* (the nuclear gene that codes for the enzyme enoyl-ACP reductase) were the chosen targets in the apicoplast and nuclear genomes, respectively. Similar-sized probes for these genes were prepared by end labeling the gel-purified PCR products amplified with primers (Microsynth, Switzerland) 5′-CCCCAGATCTATGGAGAAAGAAGAACAAGATGCATC-3′ and 5′-CGTGCTAAGCTTTTATTCATTTTCATTGCGATATAT-3′ for *fabI* and primers 5′-CGCGGATCCATGAATAATAAATTATTTTTAAGAA-3′ and 5′-CCGCTCGAGTTTAATTTTTTATTTCTGTTATAAT-3′ for *eftu* with T4 polynucleotide kinase (GibcoBRL) and a high specific activity [γ-^{32}P]ATP (6,000 Ci/mmol, 5 mCi/ml; NEN). The specific activities of the end-labeled probes measured by liquid scintillation counting (1409 counter; Wallac, San Francisco, CA) were of the order of 10^8 cpm/μg. The blot was hybridized with the probe for *fabI*, washed under stringent conditions, and exposed in a PhosphorImager cassette (Fujifilm Ltd.) by standard procedures. The same blot was then stripped off, checked for residual probe, hybridized with the probe for *eftu*, and exposed in a PhosphorImager cassette. Probes with similar specific activities were used. The scanned autoradiograms were quantified by using Bio-Rad Quantity One software. The intensity volume (the product of the intensity and the area) of each of the dots was obtained. Apicoplast DNA/nuclear DNA copy number ratios were obtained by calculating the ratios of the intensity volumes of the corresponding dots in the autoradiograms hybridized with *eftu* and those hybridized with *fabI*. The absolute copy numbers of the apicoplast genome could not be accurately determined due to differences in the specific activities of the *eftu* and *fabI* probes. However, relative changes in copy number ratios upon treatment with drugs, which were the parameters of interest in this study, were obtained.

Competitive PCR to quantitate apicoplast DNA/nuclear DNA ratio in treated *Plasmodium falciparum*. Quantitative competitive PCR is based on the competing amplification of a standard template and the gene in the DNA of interest (4). Hence, it is imperative that the standard DNA template have the same primer sites as the gene of interest but that on amplification it produce a PCR product either shorter or longer than the gene in the DNA preparation of interest. For this purpose, the gene of interest is typically cloned in a vector with either an insertion or a deletion within the gene but with the same primers which are to be used for the competitive PCR being used. The nuclear and plastid probes that we used for determination of the plastid DNA/nuclear DNA ratio were *fabI* (the nuclear chromosomal gene that codes for the protein enoyl-ACP reductase) and *eftu* (the apicoplast gene that codes for elongation factor Tu). These genes were amplified from total DNA with the primers (Microsynth) 5′-CCCCAGATCTATGGAGAAAGAAGAACAAGATGCATC-3′ and 5′-CGTGCTAAGCTTTTATTCATTTTCATTGCGATATAT-3′ for *fabI* and primers 5′-CGCGGATCCATGAATAATAAATTATTTTTAAGAA-3′ and 5′-CCGCTCGAGTTTAATTTTTTATTTCTGTTATAAT-3′ for *eftu*. The *fabI* PCR product (1,093 bp) was cloned into the TA cloning vector pGEMT (Promega) and the *eftu* PCR product (1,253 bp) was cloned into the pET28a+ vector (Novagen) by using the restriction sites in the primers (10; S. Kanagaraj and N. Surolia, unpublished data). The deletion mutant of *fabI* (Δ*fabI*-pGEMT) was made by digestion of the *fabI*-pGEMT clone with the restriction enzyme NheI (which cuts the *fabI* gene at base positions 805 and 1084 but which does not cut the pGEMT vector), followed by gel purification, ligation with T4 DNA ligase, and transformation into DH5α competent cells. The deletion mutant of *eftu* (Δ*eftu*-pET28a+) was made by digestion of *eftu*-pET28a+ with the restriction enzyme DraI (which cuts the *eftu* gene at base positions 404, 495, 746, 767, 822, and 1076 but which does not cut the pET28a+ vector), followed by gel purification, ligation, and transformation into DH5α competent cells. PCR with the deletion mutant templates Δ*fabI*-pGEMT and Δ*eftu*-pET28a+ and with the primers used for cloning yielded PCR products which were 279 bp and 672 bp shorter, respectively (i.e., 814 bp and 581 bp, respectively) than the original clones and, therefore, the genes of interest. PCR with the *eftu*-specific primers along with the Δ*eftu*-pET28a+ clone and total genomic DNA yielded two bands of 1,253 bp and 581 bp, and PCR with the

TABLE 1. Inhibitors of various processes occurring in the malaria parasite

Inhibitor	Enzyme and/or process inhibited	Subcellular location of target	Reference(s)
Rifampin	Prokaryotic RNA polymerase	Apicoplast (predicted)	21
Clindamycin	Prokaryotic protein synthesis	Apicoplast (predicted)	21
Chloramphenicol	Prokaryotic protein synthesis	Apicoplast (predicted)	21
Tetracycline	Prokaryotic protein synthesis	Apicoplast, mitochondrion (?) (predicted)	21
Haloxyfops	Acetyl-CoA[a] carboxylase, type II fatty acid synthesis	Apicoplast implied by similarity to *Toxoplasma*	21
Fluazifops	Acetyl-CoA carboxylase, type II fatty acid synthesis	Apicoplast implied by similarity to *Toxoplasma*	21
Quizalofops	Acetyl-CoA carboxylase, type II fatty acid synthesis	Apicoplast implied by similarity to *Toxoplasma*	21
Cerulenin	Fab B/F, type II fatty acid synthesis	Apicoplast (predicted)	29, 31
Triclosan	Fab I, type II fatty acid synthesis	Apicoplast	29
Succinyl acetone	δ-Aminolevulinic acid dehydratase, heme synthesis	Apicoplast (predicted)	28
Acifluorfen	Protoporphyrinogen oxidase, heme synthesis	Mitochondrion implied by similarity to *Toxoplasma*	16, 21
Cycloheximide	Eukaryotic protein synthesis	Cytosol (predicted)	3
Chloroquine	Heme polymerization	Food vacuole	24
Radicicol	Hsp90, ATP citrate lyase implied	Cytosol	11, 26

[a] Acetyl-CoA, acetyl coenzyme A.

fabI-specific primers along with the Δ*fabI*-pGEMT clone and total genomic DNA yielded two bands of 1,093 bp and 814 bp, both of which could be easily distinguishable by standard agarose gel electrophoresis.

Competitive PCR was conducted with genomic DNA isolated from parasites obtained by saponin lysis of parasite-infected red blood cell cultures either untreated or treated with one of the following for 72 h: 100 nM cycloheximide, 100 nM chloroquine, 10 μM clindamycin, 10 μM triclosan, 10 μM cerulenin, 25 μM NAS-91, 500 μM haloxyfops, 1 mM succinyl acetone, 100 μM tetracycline, or 1 μM diphenylene iodonium (DPI). Genomic DNA was isolated from the parasites by using a QIAamp genomic DNA isolation kit (QIAGEN), following the manufacturer's instructions. The PCR conditions used were 1 cycle at 95°C for 5 min and then 25 cycles of 95°C for 1 min, 50°C for 30 s, and 72°C for 45 s, followed by 1 cycle at 72°C for 5 min. The standard DNA templates used for the competitive PCRs for *fabI* and *eftu* were various dilutions of Δ*fabI*-pGEMT and Δ*eftu*-pET28a+, respectively, in calf thymus DNA. The primers used did not amplify any PCR product from calf thymus DNA, as verified by agarose gel electrophoresis. There were no significant changes in the efficiencies of amplification of *fabI*-pGEMT and Δ*fabI*-pGEMT or of *eftu*-pET28a+ and Δ*eftu*-pET28a+. The PCR products obtained following competitive PCR were resolved by agarose gel electrophoresis and stained with ethidium bromide, and the amounts of the two PCR products in each case were assessed by densitometry (Bio-Rad Quantity One software). The copy number of the genomic DNA was quantified as the copy number of the standard template in the reaction when both the PCR products were of the same intensity (see Fig. 4).

Effects of triclosan and clindamycin on incorporation of [^{14}C]acetate into fatty acids in *P. falciparum*. Parasite cultures (25 ml) that had been pretreated with triclosan (10 μM) for 2 h or with clindamycin (10 μM) for 24 h or 72 h were resuspended in 5 ml of complete medium while retaining the same concentration of the inhibitor. [1,2-^{14}C]acetate (50 μCi/ml [60 mCi/mmol] sodium acetate; NEN) was added. After 2 h, the parasites were isolated, washed thoroughly with phosphate-buffered saline, lysed, sonicated, spotted onto a Whatman 3MM paper disk, and counted in scintillation fluid for estimation of the level of incorporation of [^{14}C]acetate into fatty acids (18). The pretreated cultures were also analyzed for inhibition of growth by microscopy and by monitoring the incorporation of [^{35}S]methionine (10 mCi/ml, 1,175 Ci/mmol; NEN) into the proteins.

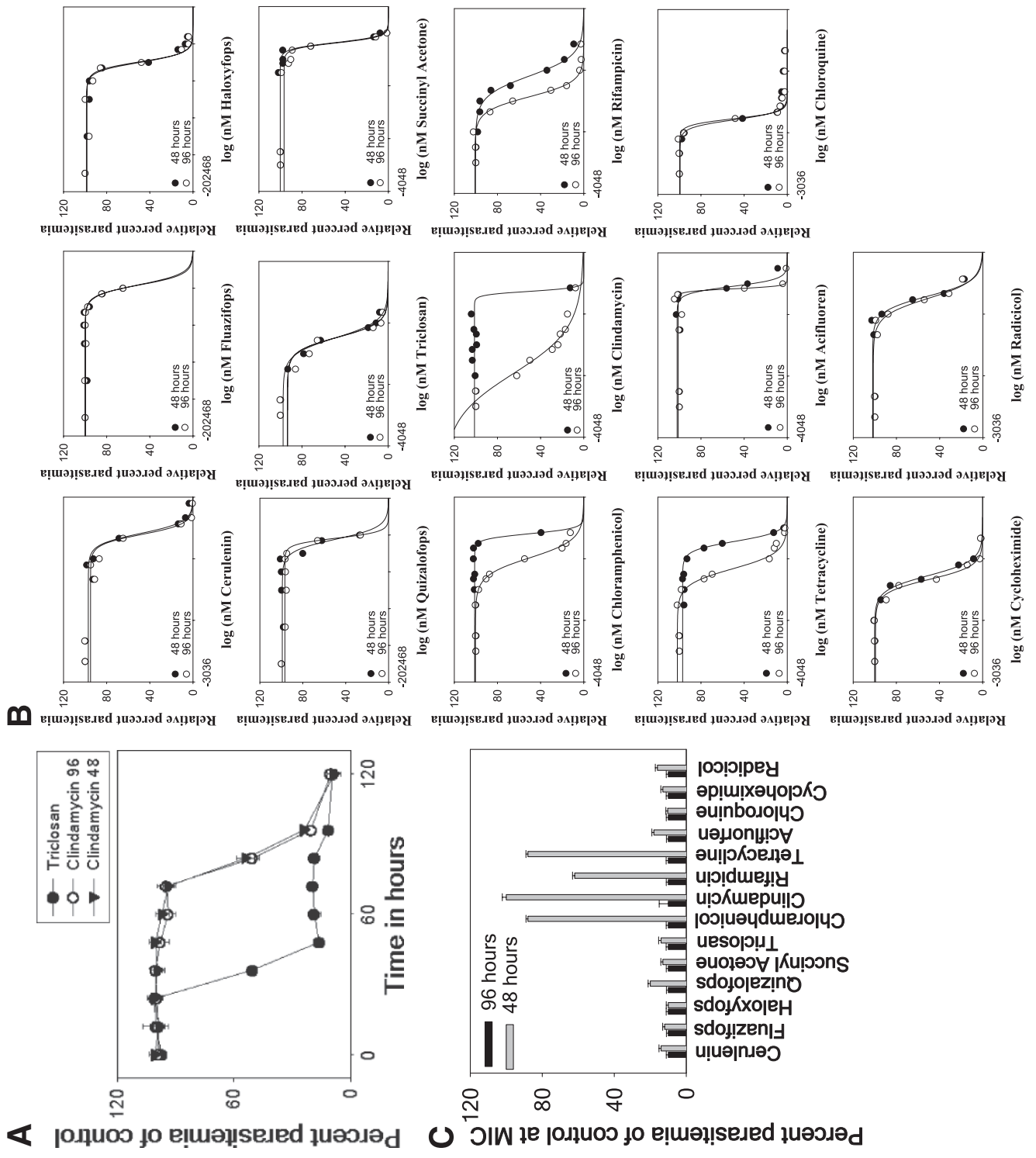
Effect of lipoic acid on inhibition of malaria parasites by triclosan and chloramphenicol. The parasite cultures were tested for their susceptibilities to triclosan and chloramphenicol by the [^{3}H]hypoxanthine uptake assay outlined earlier in the presence of 100 μM (the highest noninhibitory concentration of lipoic acid, as determined by [^{3}H]hypoxanthine uptake [data not shown]) of *R*-lipoic acid, *S*-lipoic acid, the methyl ester of octanoic acid, or the methyl ester of palmitoic acid. The IC_{50}s of the inhibitors were calculated in each of these cases.

Methyl esters of palmitic acid and octanoic acid were synthesized by reflux in 150 ml dry methanol containing 2 ml concentrated sulfuric acid until the respective acids were no longer detected by thin-layer chromatography. After the mixture was cooled, the solvent was partially evaporated under reduced pressure and the solution was neutralized with 10% sodium carbonate. The mixture was then extracted with diethyl ether (100 ml, three times). The organic phases were combined, washed three times with 100 ml, dried over magnesium sulfate, and concentrated under reduced pressure. The residue was purified by column chromatography.

Statistical methods. The two-tailed Student *t* test was used to verify the statistical significance of the differences in the percent parasitemia measured in the growth inhibition assay, as well as that of the difference in the copy number ratios obtained by quantitative hybridization and competitive quantitative PCR.

RESULTS

We first examined the kinetics of death invoked by inhibitors of various processes occurring in different subcellular organelles of the malaria parasite (Table 1) by morphological examination as well as by [^{3}H]hypoxanthine uptake. Treatment with 5 μM clindamycin or 50 μM chloramphenicol did not produce a significant decrease in parasitemia during the first cycle of asexual reproduction of the culture. However, parasitemia decreased sharply at about 84 h (Fig. 1A), which coincides with the late trophozoite stage of the second cycle of asexual reproduction (Fig. 1A). Importantly, the trend remained the same whether the cultures were incubated with the drug throughout the duration of the experiment or only during the first 48 h (i.e., roughly the first cycle of asexual reproduction), confirming delayed death as the cause of the antiparasitic effects of these antibiotics (Fig. 1A). The concentrations of these antibiotics for this experiment were chosen to be manyfold higher than those required for inhibition of parasite growth, yet no effect was seen at 48 h. Lower concentrations of the antibiotics in the range of their IC_{50}s also brought about a similar effect (data not shown). This confirmed the delayed actions of these antibiotics on parasite growth. Treatment with 50 μM tetracycline and 5 μM rifampin also resulted in a sharp decrease in parasitemia only at about 84 h, although there was a modest decrease in the parasitemia during the first cycle, also. This effect in the first cycle is probably due to inhibition of prokaryotic protein synthesis in the mitochondrion, in addition to that in the apicoplast. Lower concentrations of tetracycline and rifampin also inhibited parasite growth only in the second cycle, without affecting growth at 48 h (data not shown). That tetracycline and rifampin did bring about delayed death was also clear from the growth inhibition curves, which indicated that the concentrations of these compounds required to inhibit parasite growth at 48 h and 96 h were significantly


A
Percent parasitemia of control
Triclosan
Clindamycin 96
Clindamycin 48
Time in hours
B
Relative percent parasitemia
48 hours
96 hours
log (nM Cerulenin)
log (nM Fluazifops)
log (nM Haloxyfops)
log (nM Quizalofops)
log (nM Succinyl Acetone)
log (nM Triclosan)
log (nM Chloramphenicol)
log (nM Clindamycin)
log (nM Rifampicin)
log (nM Tetracycline)
log (nM Acifluoren)
log (nM Chloroquine)
log (nM Cycloheximide)
log (nM Radicicol)
C
Percent parasitemia of control at MIC
96 hours
48 hours
Cerulenin
Fluazifops
Haloxyfops
Quizalofops
Succinyl Acetone
Triclosan
Chloramphenicol
Clindamycin
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Tetracycline
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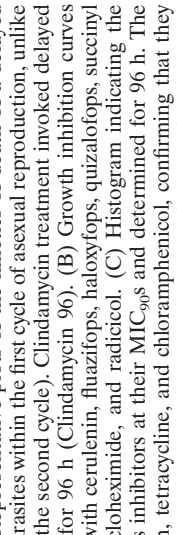

FIG. 1. Inhibition of malaria parasite by inhibitors of apicoplast and nonapicoplast functions. (A) Representative plots of the kinetics of death of a delayed death-invoking agent, clindamycin, and an agent invoking rapid death, triclosan. Triclosan ablated the parasites within the first cycle of asexual reproduction, unlike clindamycin, which invoked parasite death only after 84 h (coinciding with the late trophozoite stage of the second cycle). Clindamycin treatment invoked delayed death irrespective of whether the parasites were treated for the first 48 h only (Clindamycin 48) or for 96 h (Clindamycin 96). (B) Growth inhibition curves determined by hypoxanthine uptake of parasites at 48 h and 96 h following treatment for the first 48 h with cerulenin, fluazifops, haloxyfops, quizalofops, succinyl acetone, triclosan, chloramphenicol, clindamycin, rifampin, tetracycline, acifluorfen, chloroquine, cycloheximide, and radicicol. (C) Histogram indicating the relative percent parasitemia measured at 48 h and at 96 h in parasite cultures treated with the various inhibitors at their MIC_{90}s and determined for 96 h. The parasitemia was significantly higher at 48 h than at 96 h in cultures treated with clindamycin, rifampin, tetracycline, and chloramphenicol, confirming that they invoke delayed death. The statistical significance of the difference in percent parasitemia was confirmed by the two-tailed Student *t* test ($P < 0.05$).

different (Fig. 1B; Table 2). Therefore, the primary target of these compounds seemed to be the apicoplast. Figure 1C depicts the differences in the growth of parasites treated for 48 h and for 96 h with clindamycin, chloramphenicol, rifampin, and tetracycline at their MIC_{90}s, as determined by [^{3}H]hypoxanthine uptake. While these inhibitors reduced the parasitemia level to 10% after 96 h, they did not have much of an effect on the parasites at 48 h, thus reiterating delayed death as the mode of death induced by these antibiotics. Thus, inhibition of an apicoplast process such as protein synthesis does bring about delayed death in *Plasmodium falciparum*, too, as in *Toxoplasma gondii*.

On the contrary, agents with nonapicoplast targets, such as chloroquine, amodiaquine, clotrimazole, radicicol, cycloheximide, glyphosate, DPI, and acifluorfen, as well as agents like triclosan, thiolactomycin, cerulenin, NAS-91, and the fops series of inhibitors, which target fatty acid synthesis in the apicoplast, and succinyl acetone, which inhibits heme biosynthesis in this organelle, invoked death itself at about 36 h (Fig. 1A), which corresponds to the trophozoite stage of the first cycle of asexual reproduction. Parasites incubated with these inhibitors were killed before the culmination of the first cycle of asexual reproduction, and hence, there was no reinvasion of new host cells. The IC_{50}s determined for cultures incubated with these agents for 48 and 96 h showed no statistically significant difference (Student's *t* test, $P < 0.01$), indicating the absence of delayed death in treatments with these agents (Fig. 1B; Table 2). The percent parasitemias of cultures treated with these inhibitors at their MIC_{90}s for 48 h and 96 h were the same (~10%), thus confirming rapid death (Fig. 1C).

Inhibitors of fatty acid synthesis and heme synthesis therefore seem to elicit rapid death. However, the ultimate proof of rapid death rests with the demonstration of an unaltered apicoplast genome copy number relative to the nuclear genome copy number, i.e., no loss of the apicoplast. We used quantitative hybridization and competitive PCR to determine the apicoplast genome copy number (and, hence, to determine the presence of the apicoplast) in control and treated cultures.

We used quantitative hybridization analysis to examine the reduction, if any, in the copy number of the apicoplast genome upon treatment with clindamycin and chloramphenicol and also to confirm the absence of the delayed death phenotype with triclosan treatment. As shown in Fig. 2, the apicoplast genome is present at ~0.91 copies per haploid nuclear genome in the untreated parasites. On treatment with clindamycin, the plastid genome copy number was specifically reduced to ~0.65 copies after 48 h and was further reduced to ~0.42 copies after 96 h, indicating that the delayed death invoked by clindamycin treatment occurs subsequent to apicoplast loss. The partial apicoplast effect after 48 h of treatment with clindamycin which we observed is similar to that reported earlier for *Toxoplasma gondii* (2). It is expected that with continuous treatment with clindamycin, parasites that did not lose the apicoplast in the first 48 h (probably either due to the inherent efficacy of clindamycin or due to the absence of absolute synchrony during clindamycin treatment) would do so by 96 h, and hence, the effect at 96 h would be greater than that at 48 h.

Treatment with triclosan, however, showed a decline in both nuclear and plastid genome copy numbers in parallel, indicating that the parasites per se were ablated within the first cycle

TABLE 2. IC_{50}s and MIC_{90}s of various inhibitors determined by hypoxanthine uptake of malaria parasite cultures at 48 h and 96 h following treatment for the first 48 h

Inhibitor	IC_{50} (μM) (error) at:		MIC_{90} (μM) (error) at:	
	48 h	96 h	48 h	96 h
Cerulenin	15.1 (1.1)	16.9 (1.1)	64.9 (2.2)	52.3 (9.6)
Fluazifops	1,548.8 (110)	1,479.1 (115.0)	6,918.3 (858.1)	5,754.4 (631.5)
Haloxyfops	107.1 (10.9)	97.7 (10.3)	346.1 (1.0)	346.0 (1.6)
Quizalofops	575.4 (51.8)	676.1 (40.2)	3,090.3 (823.0)	1,948.8 (398.1)
Succinyl acetone	17,782.8 (1,102.3)	17,378.0 (1,092.4)	58,884.4 (1,148.1)	51,286.1 (3,391.2)
Triclosan	1.1 (0.01)	1.2 (0.1)	15.8 (3.3)	10.2 (1.7)
Chloramphenicol	416.8 (10.3)	11.2 (1.2)	1,023.3 (120.2)	190.5 (10.2)
Clindamycin	288.4 (21.8)	0.0013 (0.0005)	537.0 (13.8)	6.0 (0.2)
Rifampin	4.2 (0.12)	0.19 (0.011)	109.6 (2.5)	2.0 (0.04)
Tetracycline	134.9 (10.5)	2.19 (0.1)	562.3 (11.7)	26.9 (1.5)
Acifluorfen	524.8 (31.2)	457.1 (40.3)	871.0 (90.2)	691.8 (51.8)
Chloroquine	0.0048 (0.0015)	0.0042 (0.0011)	0.011 (0.0006)	0.011 (0.0003)
Cycloheximide	0.15 (0.01)	0.10 (0.03)	0.73 (0.11)	0.48 (0.09)
Radicicol	7.2 (1.2)	5.6 (0.7)	46.8 (5.2)	60.2 (6.3)

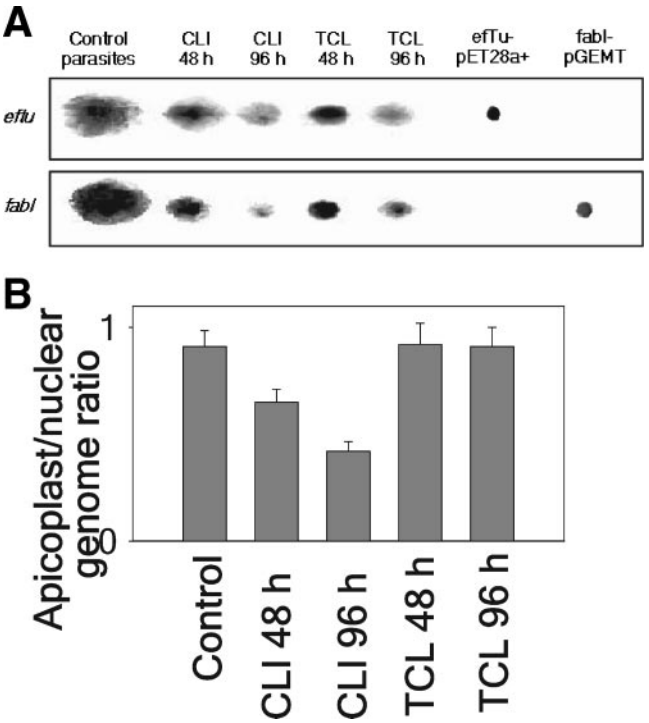

FIG. 2. Effects of triclosan and clindamycin on apicoplast genome copy number. Autoradiograms were obtained by hybridization of the dot blots with probes specific for *eftu* and *fabI*, respectively. Dots, from left to right, respectively: genomic DNA from untreated parasites, from parasites treated with clindamycin (CLI) for 48 h, parasites treated with clindamycin for 96 h, parasites treated with triclosan (TCL) for 48 h, parasites treated with triclosan for 96 h, DNA from pET28a-EF-Tu, and DNA from pGEMT-FabI. No nonspecific hybridization of *fabI* and *eftu* was observed (no signals were obtained for the *eftu*-pET28a clone with the *fabI* probe or for the *fabI*-pGEMT clone with the *eftu* probe). Quantitative analysis of the dot blots hybridized with the probes specific for a nuclear gene (*fabI*) and an apicoplast gene (*eftu*) shows a relative reduction in apicoplast genome copy number upon treatment with clindamycin but not upon treatment with triclosan. The statistical significance of changes in nuclear genome copy number/plastid genome copy number ratios was confirmed by the two-tailed Student *t* test ($P < 0.01$).

of growth itself. These data are suggestive of a fundamental difference in the mechanisms of action of triclosan and clindamycin and emphasize our observation that triclosan invokes rapid death and not delayed death in the malaria parasite, even though each protein targets an enzyme of the apicoplast.

While the data obtained upon quantitative hybridization analysis did provide biochemical proof for the loss of the apicoplast in clindamycin-treated parasites, it typically suffers from the disadvantages of the unequal specific activities of the probes and errors that arise from the unequal transfer and incomplete stripping of the probe. We therefore decided to accurately determine the absolute apicoplast copy numbers using quantitative competitive PCR. DNA from parasites treated for 72 h with triclosan, clindamycin, chloroquine, haloxyfops, succinyl acetone, NAS-91, cerulenin, DPI, cycloheximide, and tetracycline were subjected to quantitative competitive PCR. As shown in Fig. 3, while treatment with clindamycin and tetracycline attenuated the apicoplast copy number significantly from 1.0 to 0.4 and 0.1, respectively, treatment with triclosan, chloroquine, haloxyfops, succinyl acetone, NAS-91, cerulenin, DPI, and cycloheximide did not alter the ratio of the apicoplast genome copy number to the nuclear genome copy number. The significant difference between the apicoplast effect obtained following treatment with clindamycin and tetracycline could be due to the differences in the levels of the drugs with respect to their IC_{50}s.

We monitored the incorporation of [1,2-^{14}C]acetate into fatty acids in cultures treated with clindamycin to check whether inhibition of the housekeeping functions of the apicoplast leads to inhibition of fatty acid synthesis in the first cycle of asexual reproduction and to confirm apicoplast loss and the consequent absence of the fatty acid synthesis pathway in the second cycle of asexual reproduction. While the inhibitor of FabI, triclosan, inhibited fatty acid biosynthesis, as determined by the decreased level of incorporation of [1,2-^{14}C]acetate into fatty acids, treatment with clindamycin did not result in a decreased incorporation of [1,2-^{14}C]acetate into fatty acids in the first cycle of asexual reproduction (Fig. 4). In the second cycle of asexual reproduction, however, very little incorporation of [1,2-^{14}C]acetate into fatty acids was seen in clindamy-

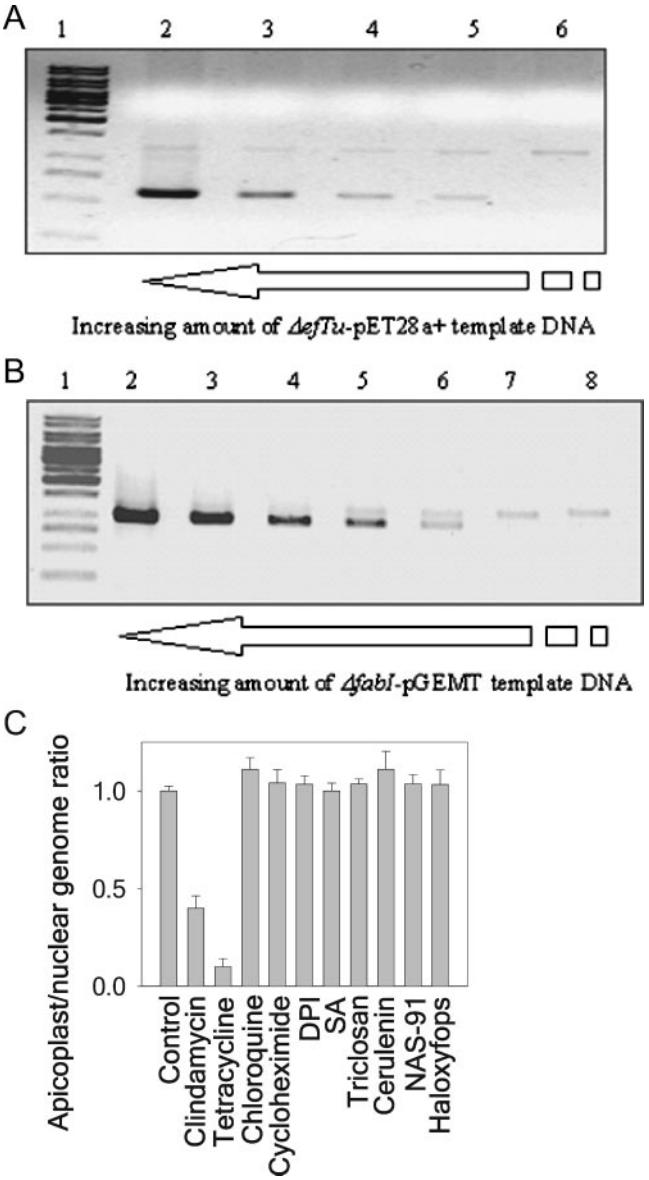

FIG. 3. Effects of inhibitors of apicoplast functions on apicoplast genome copy number. (A) Representative agarose gel pictures of competitive PCR of triclosan-treated *Plasmodium* genomic DNA with various concentrations of Δ*efTu*-pET28a+ standard DNA (lane 1, DNA molecular marker; lanes 2 to 6, various ratios of standard DNA to genomic DNA). (B) Representative agarose gel pictures of competitive PCR of triclosan-treated *Plasmodium* genomic DNA with various concentrations of Δ*fabI*-pGEMT standard DNA (lane 1, DNA molecular marker; lanes 2 to 8, various ratios of standard DNA to genomic DNA). (C) Ratios of nuclear genome copy number/apicoplast genome copy number calculated from quantitative competitive PCR show a relative reduction in the apicoplast genome copy number upon treatment with clindamycin and tetracycline but not upon treatment with triclosan, chloroquine, haloxyfops, succinyl acetone (SA), NAS-91, cerulenin, diphenylene iodonium, or cycloheximide.

cin-treated parasites, indicating apicoplast loss and a consequent absence of fatty acid synthesis (Fig. 4).

We also monitored the effect of lipoic acid on growth inhibition of the parasites by triclosan and chloramphenicol. Lipoic

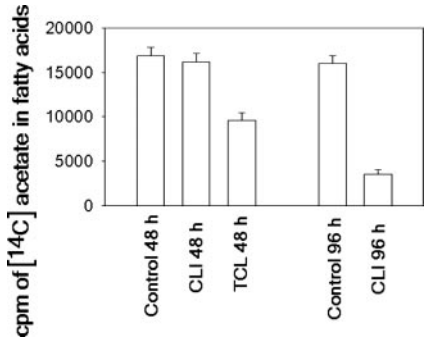

FIG. 4. Effects of inhibitors of apicoplast functions on fatty acid synthesis. [^{14}C]acetate incorporation into fatty acids is not inhibited by clindamycin in the first cycle of asexual reproduction, thus demonstrating that inhibition of an apicoplast housekeeping function like protein synthesis does not affect other apicoplast functions like fatty acid synthesis. In the second cycle, however, there is very little incorporation of [^{14}C]acetate into fatty acids, confirming apicoplast loss and the consequent absence of a functional fatty acid synthesis pathway. [^{14}C]acetate incorporation into fatty acids is reduced in the first cycle itself by the fatty acid synthesis inhibitor triclosan (TCL). CLI, clindamycin.

acid neither delayed the rapid death invoked by triclosan nor reversed the inhibition by chloramphenicol (Fig. 5).

DISCUSSION

Within a couple of years of the discovery of the apicoplast, Fichera and Roos demonstrated that the apicoplast is indispensable to the apicomplexan parasite *Toxoplasma* (2). Antibiotics that incapacitate the housekeeping functions of DNA replication, transcription, and protein synthesis of this organelle interfere with the replication of the organelle and, consequently, with its segregation during schizogony. Transient mutants that are incapable of replicating the apicoplast also share the same fate (6). Although *Toxoplasma* and *Plasmodium* have similar life cycles, while each infectious 48-h erythrocytic asexual cycle of *Plasmodium* involves only one schizogony cycle, followed by rupture of the host red blood cell, and while the merozoites generated during every schizogony cycle must invade a new host cell to continue the asexual stage of the life cycle, in *Toxoplasma* the ~48-h asexual cycle comprises several ~7-h doublings of a tachyzoite within the same host cell, followed by host cell lysis. Daughter cells of *Toxoplasma* with compromised apicoplasts are able to survive while they remain in the same host cell. However, even though they appear to be healthy and grow at a normal rate, they are unable to successfully establish a new infection and, hence, die a delayed death. Exactly what causes the delayed death phenomenon following the loss of apicoplast function still remains a mystery. It has been postulated that the apicoplast is required for replenishing the reserves of a resource presumably involved in the generation of the parasitophorous vacuole that surrounds the parasite in the host cell and is crucial to a successful host cell invasion (2, 22). In the event of such a scenario, identification of the key molecules that are synthesized by the apicoplast and that obviate a rapid death would provide insight into understanding the phenomenon of delayed death. Most of the predicted apicoplast-targeted proteins with anabolic func-

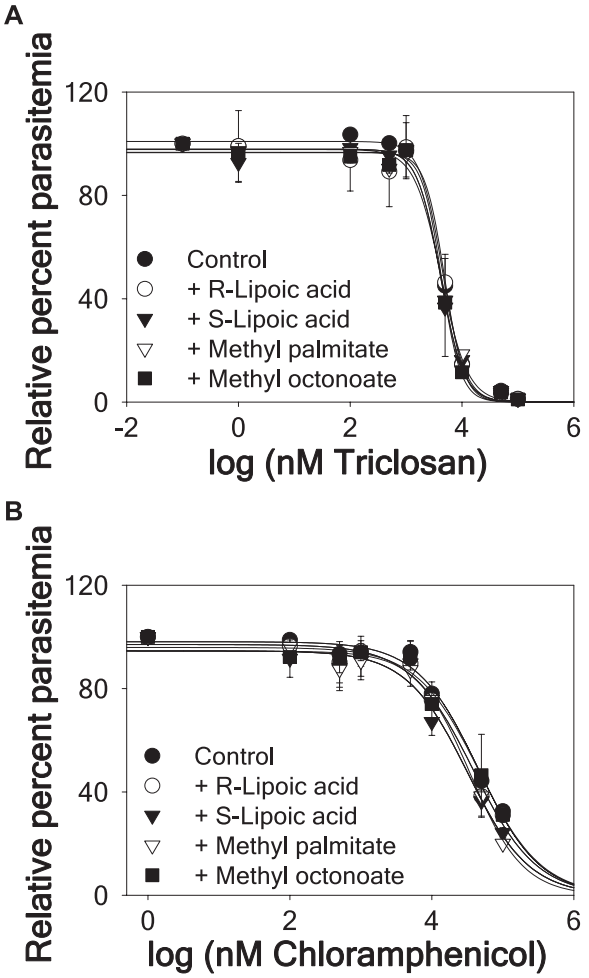

FIG. 5. Effect of lipoic acid on parasite growth inhibition by inhibitors of apicoplast functions. (A) Malaria parasite growth monitored by [^{3}H]hypoxanthine uptake assay in the presence of *R*-lipoic acid, *S*-lipoic acid, methyl octonoate, methyl palmitate, and triclosan. (B) Malaria parasite growth monitored by [^{3}H]hypoxanthine uptake assay in the presence of *R*-lipoic acid, *S*-lipoic acid, methyl octonoate, methyl palmitate, and chloramphenicol.

tions are involved in the fatty acid biosynthesis, heme biosynthesis, and non-mevalonate isopentenyl phosphate biosynthesis pathways. All these pathways involved in the production and modification of lipids and lipid-bound proteins could be required for the parasite-host cell interaction or for the generation of the parasitophorous vacuole, which are essential for successful invasion of the host cell (22). Hence, according to the existing paradigm, compromising any apicoplast function should kill the parasites by interfering with host cell invasion, potentially only via the clichéd delayed death route (22).

Our experiments demonstrate that in *Plasmodium falciparum*, too, as in *Toxoplasma gondii*, the antibiotics chloramphenicol, clindamycin, rifampin, and tetracycline invoke delayed death and kill the parasites only in the second cycle of asexual reproduction, However, host cell invasion (which includes the process of parasitophorous vacuole formation) is not the process that is crucially affected. In fact, following treatment with these antibiotics, parasites in the second asexual cycle do not progress to the schizont stage. This discrepancy in the actions of these antibiotics on *Toxoplasma* and *Plasmodium* probably stems from the occurrence of the cytosolic type I fatty acid biosynthesis pathway in *Toxoplasma* (22). It is possible that in the presence of a functional cytosolic fatty acid synthesis pathway, fatty acids synthesized by the type II fatty acid synthesis pathway resident in the plastid in *Toxoplasma* are specifically required during the process of host cell invasion. In contrast, *Plasmodium* harbors only a type II fatty acid synthesis pathway in the apicoplast, which therefore must minister to the lipid requirement of not just host cell invasion but other processes as well. Subsequently, apicoplast loss following treatment of *Plasmodium* with delayed death-invoking agents kills the parasites in the trophozoite stage of the second asexual cycle, as observed experimentally. Importantly, treatment of *Plasmodium* with inhibitors of fatty acid synthesis or heme synthesis leads to rapid death and not delayed death.

Our findings pose a question, however. If the fatty acid and heme biosynthesis pathways are so crucial to the survival of *Plasmodium* that parasites treated with inhibitors of the fatty acid and heme biosynthesis pathways do not survive to invade the next host cell, how do parasites treated with delayed death-invoking agents survive almost an entire cycle without a functional apicoplast (and, hence, a functional fatty acid synthesis or heme synthesis pathway)? This apparent paradox is resolved when it is recalled that fatty acid synthesis occurs maximally in the metabolically active trophozoite stage (29). The fatty acids synthesized in the trophozoite stage are crucially required during the trophozoite stage and are probably sufficient to last the parasite until the following invasion by the merozoite. Inhibition of fatty acid synthesis in the trophozoite stage by an inhibitor of fatty acid synthesis generates a paucity of fatty acids. The trophozoite stage fails to progress to the schizont stage, and the parasite dies a rapid death. However, all proteins involved in the functioning of the fatty acid, heme, and isoprenoid biosynthesis pathways are nucleus encoded and apicoplast targeted, and inhibition of apicoplast DNA, RNA, or protein synthesis (by clindamycin) does not interfere with fatty acid biosynthesis in the apicoplast in the first cycle of asexual reproduction (Fig. 4). Consequently, during the first 48 h of treatment with a delayed death-invoking agent, fatty acids are still synthesized, despite the inhibition of apicoplast DNA replication, transcription, or protein synthesis. This allows the survival and invasion of the next host cell. In the second cycle, in the absence of an apicoplast, fatty acids are not synthesized during the trophozoite stage of the malaria parasite, and hence, they are unable to survive and progress to the schizont stage.

In lieu of our observation that the apicoplast ministers to the parasite's requirement of lipids essential not just for host cell invasion but also during the metabolically active trophozoite stage itself, we wondered if lipoic acid, a cofactor of mitochondrial α-keto acid dehydrogenase, which is synthesized in the apicoplast, is the "key" molecule synthesized by the apicoplast. Lipoic acid is an efficient free radical scavenger and plays a pivotal role in proffering protection against oxidative insults (19, 36). In fact, Toler suggests that the apicoplast in apicomplexans, by virtue of its ability to synthesize lipoic acid, was retained as an obligate endosymbiont under evolutionary selection pressure to combat the oxidative injury generated by

mitochondrial reactive oxygen species during pyrimidine biosynthesis in the schizont stage (30). Lipoic acid is produced from octanoyl-ACP and cysteine by the apicoplast resident lipoic acid synthase (LipA). Furthermore, synthesis of the enzyme LipA, which utilizes an Fe-S cluster, is dependent on parasite heme biosynthesis. Consequently, inhibition of fatty acid synthesis or heme synthesis should deprive the parasite of lipoic acid. If parasite survival hinged on lipoic acid reserves, then the exogenous addition of lipoic acid would enable the parasite to overcome fatty acid or heme synthesis inhibition. However, the results of our experiments demonstrate that the externally administered lipoic acid is not capable of rescuing triclosan-treated parasites from rapid death (Fig. 5). Lipoic acid also could not rescue chloramphenicol-treated parasites from delayed death (Fig. 5). Our findings suggest that Toler's hypothesis (30) is an oversimplified one. While lipoic acid could be one of the essential molecules synthesized by the apicoplast, the apicoplast, in all probability, does not exist for the synthesis of one key molecule but exists for the synthesis of various molecules, many of which are essential for parasite survival.

Our study unequivocally demonstrates that although drugs which interfere with the processes of apicoplast replication, transcription, and translation lead to apicoplast loss, they do not kill the malaria parasite rapidly, as they permit other apicoplast biochemical processes essential to the survival of the parasite to proceed, thus enabling it to survive a cycle of growth. However, inhibitors of fatty acid biosynthesis and heme biosynthesis, which are essential functions of the apicoplast, like the processes mentioned above, appear to impinge more critically on the very survival of the parasite. These inhibitors kill the parasite as rapidly as inhibitors or drugs that work on targets outside the apicoplast. Thus, the machinery within the apicoplast appears to serve two distinct but related functions, i.e., "self-sustenance" and the "sustenance of the organism" as a whole. The observation of this dichotomy provides hope for the further development of drugs which inhibit processes other than those intimately linked with the self-sustenance of the apicoplast per se. Inhibitors of fatty acid synthesis, heme biosynthesis, and probably, isoprenoid synthesis, too, could very well be our best bets as therapeutic agents that can be used to combat malaria rapidly and effectively.

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